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The Western Wheatgrass Chloroplast Genome Originates in *Pseudoroegneria*

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ABSTRACT

The octoploid *Pascopyrum smithii* (Rydb.) A. Löve (StHNsXm) arose from hybrization among four diploid genera, three of which are known from genome analysis, but the diploid origin of the *Pascopyrum* chloroplast genome remains unknown. Identification of the maternal parents in the hybridizations leading to *Pascopyrum* will guide efforts to reconstruct allopolyploid germplasm from ancestral taxa. We compared the DNA sequences of a 762-base pair (bp) segment of the ndhF (nicotinamide adenine dinucleotide dehydrogenase subunit F) chloroplast gene of western wheatgrass (*Pascopyrum smithii*; StHNsXm) with that of its putative allotetraploid, i.e., *Elymus* (StH) and *Leymus* (NsXm), and diploid, i.e., *Pseudoroegneria* (St), *Hordeum* (H), *Psathyrostachys* (Ns), ancestors. To ascertain the 2x to 8x chloroplast phylogeny, the gene sequences were aligned and a phylogenetic tree was constructed by the neighbor-joining method. *Pascopyrum smithii* differed by 0 to 2 and 6 to 9 bp from its two 4x ancestors, *Elymus* and *Leymus*, respectively. *Elymus* differed by 2 and 10 to 13 bp from its two 2x ancestors, *Pseudoroegneria* and *Hordeum*, respectively. *Pascopyrum*, *Elymus*, and *Pseudoroegneria* taxa clustered together, but separately from *Leymus* and *Hordeum* taxa. The *Pascopyrum* chloroplast genome appears to have originated from the diploid *Pseudoroegneria* through the tetraploid *Elymus*. De novo synthesis of *Pascopyrum* germplasm from its tetraploid ancestors should be conducted with cognizance of the preference for *Pseudoroegneria* cytoplasm found in nature. *Leymus* differed from its diploid ancestor, *Psathyrostachys*, by 14 to 15 bp, indicating that the second unknown diploid ancestor of *Leymus* may have contributed its chloroplast DNA.

SPECIES of *Pascopyrum*, *Elymus*, *Leymus*, and *Pseudoroegneria* are important range grasses in western North America. Western wheatgrass (*Pascopyrum smithii* [Rydb.] A. Löve), the sole member of the genus (Löve, 1980), is octoploid (Gillet and Senn, 1960), although aneuploids are frequent in this species because it may propagate by rhizomes (Dewey, 1975). North American *Elymus* (**StH**) and most *Leymus* (**NsXm**) are tetraploid, while most North American *Pseudoroegneria* (**St**) are diploid. Dewey (1975) maintained that western wheatgrass is an allooctoploid hybrid between *Elymus* and *Leymus* tetraploids. *Elymus* has been designated as the genus to include all **StH** tetraploid species (Dewey, 1984). Investigators agree that one of the *Leymus* genomes originates from *Psathyrostachys* (**Ns**), but delineation of the second has been problematic (Zhang and Dvořák, 1991). Wang and Hsiao (1984) accepted **J** (= $\mathbf{E}^{b}$ of *Thinopyrum bessarabicum*) as the second *Leymus* genome on the basis of cytological (Petrova, 1970) and morphological (Löve, 1984) considerations, but more recent DNA hybridization (Zhang and Dvořák, 1991; Wang and Jensen, 1994) and genomic (Wang and Jensen, 1994) data have eliminated it as a possible *Leymus* genome. Zhang and Dvořák (1991) presented repeated-sequence hybridization data which indicated that the second genome of *Leymus* is also derived from *Psathyrostachys*. Wang and Jensen (1994), however, found insufficient numbers of trivalents in *Leymus* × *Psathyrostachys* triploid hybrids to justify this conclusion and contended that the identity of the second genome remains unknown. This genome has been designated as **Xm**, making *Leymus* **NsXm** and *Pascopyrum* **StHNsXm** (Wang et al., 1995).

On the basis of morphological traits, ecological adaptation, germination characteristics, geographical distribution, and reproductive biology, Dewey (1975) suggested that the most likely tetraploid progenitors of western wheatgrass were thickspike wheatgrass [*E. lanceolatus* (Scribn. & J.G. Smith) Gould] and beardless wildrye [*L. triticoides* (Buckl.) Pilger]. However, the thickspike wheatgrass × beardless wildrye hybrid was never made by Dewey, and its genetic similarity to western wheatgrass remains undemonstrated. Instead, amphiploids of the genomically similar *E. canadensis* × *E. dasystachys* (= *L. secalinus*) hybrid were crossed with western wheatgrass, producing progeny when the amphiploid served as the pollen parent (Dewey, 1975). All of these octoploid hybrids (western wheatgrass × amphiploid), however, exhibited meiotic abnormalities and were completely sterile.

The chloroplast genome is maternally inherited in grasses and provides a mechanism to determine the direction of hybridization in polyploid evolution. Here, we sequenced a 762-bp segment from the ndhF chloroplast gene of octoploid western wheatgrass (**StHNsXm**) from putative tetraploid (*Elymus* [**StH**], *Leymus* [**NsXm**]) and diploid (*Pseudoroegneria* [**St**], *Hordeum* [**H**], *Psathyrostachys* [**Ns**]) ancestors. Our objective was to identify the diploid origin of the chloroplast genome of *Pascopyrum*, *Elymus*, and *Leymus* polyploids. This information will guide efforts to reconstruct allopolyploid germplasm from ancestral taxa.

MATERIALS AND METHODS

Seedlings of 17 accessions (Genbank accession numbers beginning with AF) representing the genera *Pascopyrum* (8x), *Elymus* (4x), *Pseudoroegneria* (2x), *Hordeum* (2x), *Leymus* (4x), and *Psathyrostachys* (2x) were included here (Table 1). Young leaves from two greenhouse-grown plants per accession were harvested and immersed in liquid nitrogen immedi-

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Table 1. Species, ploidy, genomic formulae (Wang et al., 1995), cultivar or accession identifiers, origins, and Genbank accession numbers for ndhF nucleotide sequences.

Species	Ploidy	Genomic formula	Cultivar or accession identifier	Origin	Genbank accession number
Pascopyrum smithii	8x	StHNsXm	Rodan	Morton Co., ND	AF056170
			Flintlock	Nebraska–Kansas	AF056169
			Barton	Barton Co., KS	AF056174
			Atkins-172	Utah	AF056178
			Arriba	Flagler, CO	AF056172
			R-9-1—5	Green River, WY	AF056171
			Walsh	Alberta–Saskatchewan	AF056177
			Atkins-142	Arizona	AF056173
			Rosana	Forsyth, MT	AF056175
			EPC-8	Vernal, UT	AF056176
Elymus wawawaiensis	4x	StH	Secar	Lewiston, ID	AF056168
Elymus lanceolatus	4x	StH	Critana	Havre, MT	AF056166
Pseudoroegneria spicata	2x	St	P-5	unknown	AF056166
Leymus triticoides	4x	NsXm	Acc:642	Jamieson, OR	AF056180
Leymus cinereus	4x	NsXm	Trailhead	Roundup, MT	AF056165
Hordeum bogdanii	2x	H	PI 531760	Xinjiang, China	AF056179
Hordeum vulgare	2x	I	Steptoe	–	U22002
Psathyrostachys juncea	2x	Ns	Bozoisky-Select	Kazakhstan	AF056167
Poa pratensis					U21980
Avena sativa					U21999
Piptatherum racemosum					U21924

ately prior to DNA isolation. The two plants from each accession served as replicates. Total DNA was isolated by a modification of the CTAB extraction method (Lassner et al., 1989; Williams et al., 1993). The 3′ end of the chloroplast ndhF gene (~830 bp) was amplified with 1318F and 2110R primers (Olmstead and Sweere, 1994). DNA was amplified in 100-μL reactions containing 10 m*M* Tris-HCl (pH 9.0), 50 m*M* KCl, 0.1% (v/v) Trition X-100, 0.25 m*M* dNTP, 0.86 p*M* ndhF 2110R primer, 0.42 p*M* ndhF 1318F primer, 2 m*M* $MgCl_2$, and 50 ng template DNA. Samples were preheated at 93°C for 3 min, and 2.5 U Taq DNA polymerase (Promega, Madison, WI) was added. Thirty cycles (35 s at 93°C, 35 s at 51°C, 2.5 min at 72°C) of amplification were followed by a 10-min incubation at 72°C. Size and purity of the amplified DNA was verified by agarose gel electrophoresis (Sambrook et al., 1989). The PCR products were purified with Promega Wizard PCR Preps DNA Purification System according to the manufacturer's instructions. DNA concentration was determined on a TKO 100 mini-fluorometer using Hoescht 33258 according to manufacturer's instructions (Hoefer, San Francisco, CA).

DNA sequence analysis was performed at the Utah State University Biotechnology Center on an ABI 373 autosequencer (Perkin-Elmer Applied Biosystems, Foster City, CA) using AmpliTaq or AmpliTaq FS polymerase and the dye-terminator cycle sequencing kit according to manufacturer's protocols. Both strands of each fragment were sequenced using three forward and two reverse primers. The reverse primers were the 1655R and 2110R primers designed to sequence the tobacco (*Nicotiana tabacum* L.) ndhF gene (Olmstead and Sweere, 1994). The 1318F forward primer designed by Olmstead and Sweere (1994) was also used. Two other forward primers, specific for monocot ndhF gene sequences were designed. The 1602F primer, 5′-CC(G/T)CATGAAAC-GGGAAATAC-3′, corresponded to bp 257 to 276 of the sequences reported here and bp 1602 to 1621 of the tobacco sequence (Genbank accession #TOBCPNDHF; Olmstead et al., 1993). The 1821F primer, 5′-TT(T/G/C)GGTT(C/T)TAT-TCATAGCATA-3′ corresponded to bp 497 to 516 of the analyzed sequences and bp 1821 to 1840 of the tobacco sequence.

DNA sequences were aligned by the PILEUP and LINEUP functions of the Wisconsin Package (Devereux, 1994). Phylogenetic trees were generated by the neighbor-joining distance method (Saitou and Nei, 1987) of the MEGA program (Kumar et al., 1993). Bootstrap values (based on 500 replications) were generated by MEGA. Distances among nucleotide sequences were generated by the Jukes-Cantor distance method (Jukes and Cantor, 1969). The method gives the maximum likelihood estimate of the number of nucleotide substitutions between two sequences. Our sequences met the criteria for the Jukes-Cantor method, where the number of nucleotide substitutions per site (d) was ≤ 0.3 and the transition/transversion ratio was < 2 (Nei, 1991; Kumar et al., 1993). We used the p parameter (Kumar et al., 1993), the proportion of amino acid sites at which sequences of two accessions differ, to estimate the genetic distance between their deduced amino acid sequences. Oat (*Avena sativa* L.), Kentucky bluegrass (*Poa pratensis* L.), and ricegrass [*Piptatherum racemosum* (Smith) Barkw.] served as outgroups. Sequences for these accessions as well as *Hordeum vulgare* L. were obtained from Genbank (Genbank accession numbers beginning with U) (Table 1).

RESULTS AND DISCUSSION

Several plastid DNA sequences have been used to develop phylogenies in plants including the coding regions of the chloroplast rbcL (gene for ribulose bisphosphate carboxylase–oxygenase large subunit) and ndhF genes and the intron and intergenic spacer regions of the trnL (gene for leucine tRNA) gene (Taberlet et al., 1991; Olmstead and Sweere, 1994; Clark et al., 1995). The ndhF coding region is more variable than the rbcL (Olmstead and Sweere, 1994; Clark et al., 1995), due in part to the greater length of ndhF (>2000 bp vs. 1400 for the rbcL). Among the Poaceae, Clark et al. (1995) found 60% of the variability in the 3′ third of the ndhF sequence. Because Triticeae species being examined were closely related, we focused our sequence analysis on the 3′ third of the ndhF chloroplast gene. As in other species from the Poaceae family, the most variable region of the Triticeae ndhF was at the 3′ end of the gene, especially from bp 500 to 600 (corresponding to bp 1860–1960 of the tobacco gene). Although insertions or deletions of up to 1 to 5 codons were identified among Poaceae ndhF sequences (Clark et al., 1995), none were found among the sequences we examined.

Sequences for the two individuals of each of the 18 Triticeae accessions were identical, indicating that the accessions were largely uniform for this sequence and that no point mutations were introduced during amplification. However, length differences have been found between ndhF genes of Triticeae grasses and non-Triticeae grasses, e.g., rice (*Oryza sativa* L.) (Clark et al., 1995), and dicots, e.g., tobacco (Olmstead and Sweere, 1994). The 762-bp sequence analyzed here codes for 253 amino acids, with the open reading frame beginning at bp 2. The sequence is A-T rich. The 'Rodan' western wheatgrass sequence, for example, is 69% AT.

Jukes-Cantor distances, for nucleic acid sequences, and *p*-distances, for deduced amino acid sequences, i.e., the number of differences per number of sites analyzed, were calculated for the 21 accessions. Nucleotide substitutions were found at 30 of the 762 positions (27 of the 253 codons) among the 18 Triticeae accessions. These 30 mutations consisted of 23 single-base, two double-base (bp 548–550; 587–589), and one triple-base (bp 575–577) substitution. Multiple nucleotide substitutions were found at one position within the 18 Triticeae accessions and at six additional positions among the Triticeae accessions and the outgroups. Transitions (substitutions of a purine for a purine or a pyrimidine for a pyrimidine) accounted for 46% of all nucleotide substitutions among the 18 Triticeae accessions, thus numbers of transitions and transversions (substitutions of a purine for a pyrimidine or a pyrimidine for a purine) were about equal. When all 21 accessions (including outgroups) were considered, nucleotide substitutions were found at 129 (17%) of all positions in 98 codons. Single, double, and triple-base substitutions were found in 71, 23, and 4 codons, respectively, in the 21-accession data set.

Among the 18 Triticeae accessions, nonsynonymous nucleotide substitutions resulted in 14 (6%) amino acid substitutions. Nonsynonymous substitutions occurred at 64 (25%) additional positions between the Triticeae accessions and the outgroups. Multiple amino acid substitutions were found at one position within the 18 Triticeae accessions and at eight additional positions between the Triticeae accessions and the outgroups.

Three different sequences were found among the 10 western wheatgrass accessions examined and were assigned to three different groups accordingly. Group 1 includes cultivars Flintlock (origin: central and southwestern Nebraska and northwestern Kansas), Barton (origin: Barton Co., KS), Arriba (origin: Flagler, CO), Walsh (origin: southern Alberta and southwestern Saskatchewan), Rodan (origin: Morton Co., ND), and the accessions Atkins-172 (origin: Utah) and R-9-1—5 (origin: Green River, WY); Group 2 includes the cultivar Rosana (origin: Forsyth, MT) and the accession Atkins-142 (origin: Arizona); Group 3 includes only the accession EPC-8 (origin: Vernal, UT). Groups 1 and 2 both include materials from each side of the Continental Divide. Groups 1 and 2 differed by two base pairs, Groups 2 and 3 by one base pair, and Groups 1 and 3 by three base pairs. The sequence of Group 2 was identical to the sequence of *E. lanceolatus/wawawaiensis*. Deduced amino acid sequences of Group 1 differed by two moieties from sequences of Groups 2 and 3 and the two *Elymus* accessions. Thus, 2 of the 3 base pair differences

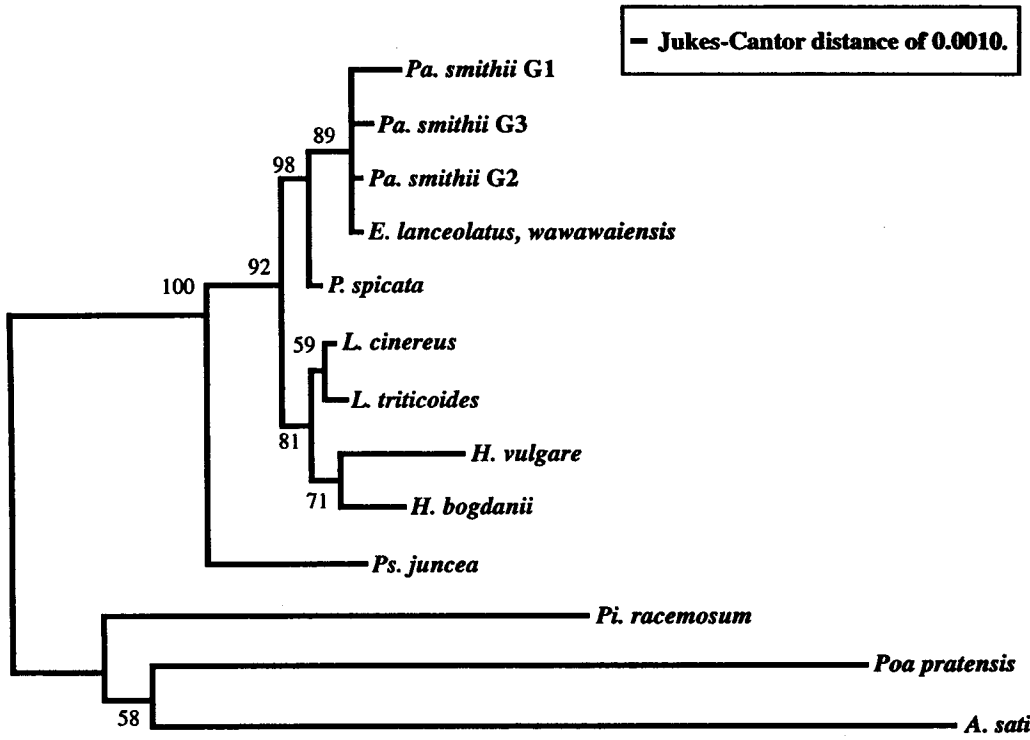

Fig. 1. Neighbor-joining phylogenetic tree for ndhF DNA sequences. *Poa pratensis*, *Avena sativa*, and *Piptatherum racemosum* served as outgroups. Numbers at the branch points give the bootstrap confidence values for 500 replications. Values greater than 90 indicate statistically significant groupings of accessions at that branch node (Kumar et al., 1993). Genetic distances calculated according to Jukes and Cantor (1969).

Table 2. Comparison of DNA nucleotide and deduced amino acid sequences for the chloroplast gene, ndhF. The Jukes-Cantor distances among the DNA sequences (above diagonal) and *p*-distances among the deduced amino acid sequences (below diagonal) were calculated using the MEGA program (Kumar et al., 1993). Identical sequences within a species were grouped. *Pascopyrum smithii* Group 1 includes cultivars Rodan, Flintlock, Barton, Arriba, and Walsh, as well as accessions Atkins-172 and R-9-1—5. *Pa. smithii* Group 2 includes the cultivar Rosana and the accession Atkins-142. *Pa. smithii* Group 3 consists solely of the accession EPC-8.

	Pascopyrum smithii												
	Group 1	Group 2	Group 3	*Leymus cinereus*	*Leymus triticoides*	*Psathyrostachys juncea*	*Hordeum vulgare*	*Hordeum bogdanii*	*Elymus* spp.	*Pseudoroegneria spicata*	*Poa pratensis*	*Avena sativa*	*Pipthatherum racemosum*
Pa. smithii G1		0.003	0.004	0.011	0.012	0.021	0.020	0.016	0.003	0.005	0.084	0.091	0.066
Pa. smithii G2	0.008		0.001	0.008	0.009	0.019	0.017	0.013	0.000	0.003	0.081	0.088	0.063
Pa. smithii G3	0.008	0.000		0.009	0.011	0.020	0.019	0.015	0.001	0.004	0.082	0.090	0.065
L. cinereus	0.020	0.016	0.016		0.001	0.019	0.012	0.008	0.008	0.005	0.081	0.085	0.060
L. triticoides	0.024	0.020	0.020	0.004		0.020	0.011	0.007	0.009	0.007	0.082	0.087	0.062
Ps. juncea	0.036	0.032	0.032	0.024	0.028		0.027	0.024	0.019	0.016	0.082	0.085	0.063
H. vulgare	0.036	0.032	0.032	0.016	0.012	0.040		0.012	0.017	0.015	0.090	0.094	0.060
H. bogdanii	0.024	0.020	0.020	0.004	0.000	0.028	0.012		0.013	0.011	0.087	0.091	0.065
Elymus spp.	0.008	0.000	0.000	0.016	0.020	0.032	0.032	0.020		0.003	0.081	0.088	0.063
P. spicata	0.012	0.008	0.008	0.008	0.012	0.024	0.024	0.012	0.008		0.078	0.085	0.060
Poa pratensis	0.127	0.123	0.123	0.115	0.111	0.115	0.127	0.115	0.123	0.115		0.103	0.081
Avena sativa	0.130	0.127	0.127	0.123	0.123	0.123	0.134	0.123	0.127	0.119	0.158		0.094
Pi. racemosum	0.099	0.095	0.095	0.087	0.091	0.083	0.095	0.091	0.095	0.087	0.127	0.130	

found within western wheatgrass were reflected in the deduced amino acid sequence.

The neighbor-joining tree for nucleotide sequence (Fig. 1) grouped the sequences for *Secar* (*E. wawawaiensis*) and Critana (*E. lanceolatus*), which were identical, with sequences of 10 western wheatgrass accessions (bootstrap value of 89%). This means that these 12 accessions clustered together and apart from all other accessions in 89% of the 500 trees constructed from random samples of the data. Jukes-Cantor distances among the 12 accessions ranged from $d = 0.000$ to 0.004 (Table 2). These data support the contention that the western wheatgrass chloroplast was inherited from the *E. lanceolatus* tetraploid.

An accession of *Pseudoroegneria spicata* clustered with the 12-accession group with a bootstrap-value of 98% (Fig. 1). Jukes-Cantor distances between *P. spicata* and these 12 accessions ranged from $d = 0.003$ to 0.005. These data support the contention that the western wheatgrass and *E. lanceolatus* chloroplasts originated in the *Pseudoroegneria* diploid.

Leymus cinereus and *L. triticoides* accessions clustered separately from the *Elymus*, *Pascopyrum*, and *Pseudoroegneria* accessions, indicating that a tetraploid *Leymus* species likely did not donate its chloroplast to *Pascopyrum* (Fig. 1). *Leymus* accessions grouped with *H. bogdanii* (**H**) and *H. vulgare* (**I**). Jukes-Cantor distances between the *Leymus*/Hordeum cluster and the *Elymus*/*Pascopyrum*/*Pseudoroegneria* cluster were $d = 0.005$ to 0.020 (Table 2). Jukes-Cantor distances between *Leymus* and *Psathyrostachys* accessions were $d = 0.019$ to 0.020. *Psathyrostachys juncea* clustered separately from all other Triticeae species (Fig. 1).

The neighbor-joining method for deduced amino acid sequence generated a tree similar to the nucleotide-sequence tree (not shown). *Pascopyrum* and *Elymus* clustered together with a bootstrap value of 93%. *p*-Distances among these 12 accessions were 0.000 to 0.008 (Table 2). These 12 accessions clustered together with *Pseudoroegneria* (bootstrap value = 87%). Variation among the 18 Triticeae accessions was found at nine of 64 amino acid positions in the deduced sequence.

Empirical studies and computer simulations have shown that the neighbor-joining method is a highly efficient method for recovering the correct topology with a relatively small number of data points (Nei, 1991; Kumar et al., 1993). Branch-and-bound and heuristic maximum parsimony trees (not shown) were essentially similar to the neighbor-joining tree in that *Pa. smithii* clustered with *Elymus* and *Pseudoroegneria spicata*. The neighbor-joining tree was also topologically identical to a tree generated by UPGMA (unweighted pair group method using arithmetic averages). The similarity of these trees suggest that the chloroplast genomes of *Pascopyrum*, *Elymus*, and *Pseudoroegneria* are closely related. The chloroplast genome of *Leymus* appears to be distantly related to these three. As such, the *Leymus* chloroplast is not likely ancestral to the *Pascopyrum* chloroplast. While *Psathyrostachys* ($2x$) is considered a direct nuclear progenitor of *Leymus* ($4x$), their chloroplasts are less closely related than *Pseudoroegneria* ($2x$) is to *Pascopyrum* ($8x$). The *Leymus* chloroplast may have originated from the unknown ancestor carrying the **Xm** genome rather than from *Psathyrostachys*. Indeed, *H. bogdanii* (**H**) and *H. vulgare* (**I**) are more similar to *Leymus* ($d = 0.007$–0.012) than *Leymus* is to *Psathyrostachys* ($d = 0.019$–0.020) (Table 2). However, fluorescent in situ hybridization data indicate that the identity of the unknown **Xm** genome is neither **H** nor **I** (R. R-C. Wang, USDA-ARS, Logan, UT, 1998, personal communication).

Our data indicate that the *Pseudoroegneria* chloroplast genome was preferred both in speciation of *Elymus* upon hybridization of its diploid ancestors and in speciation of *Pa. smithii* upon hybridization of its tetraploid ancestors. Therefore, revised genomic designations for *Pa. smithii* and *E. lanceolatus*/*wawawaiensis* are **StHNsXm** and **StH**, respectively, where the underline indicates the genome of cytoplasmic origin. While the sequences of the 10 western wheatgrass accessions surveyed were not identical, they appear to have originated from *Pseudoroegneria* in all cases. While this preference cannot be attributed to the sequence itself, the sequence has allowed us to document a preferred pattern of inher-

itance of the chloroplast genome in the polyploid evolution of western wheatgrass. De novo synthesis of *Pascopyrum* germplasm from its tetraploid ancestors should be conducted with cognizance of the preference for *Pseudoroegneria* cytoplasm found in nature.

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